Loss of Phosphatase and Tensin Homolog Protein Expression Is an Independent Poor Prognostic Marker in Lung Adenocarcinoma

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Introduction: Phosphatase and tensin homolog (*PTEN)* has been established as a tumor suppressor gene with an important role in regulating the phosphatidylinositol-3-kinase/AKT antiapoptotic and survival pathway. The prognostic role of *PTEN* in non–small-cell lung carcinoma has not been evaluated completely in the context of other molecular information.

Methods: Tissue microarrays containing 152 resected non–small-cell lung cancer specimens were used to investigate PTEN and p53 by immunohistochemistry and *PTEN* by fluorescence in situ hybridization. DNA was isolated and subjected to mutational profiling using the Sequenom Oncocarta v1.0 panel. Clinicopathological features were correlated with PTEN expression, gene copy number, and mutation status.

Results: PTEN staining was absent in 63 (41.4%) of the cases. Significantly more squamous cell carcinomas compared with adenocarcinomas demonstrated loss of (negative) PTEN staining (26 of 44 [59%] versus 32 of 94 [34%]; $p = 0.009$). *PTEN* gene copy deletion was present in only seven of 124 evaluable cases (5.6%); all deleted cases were immunohistochemistry negative. In univariate and multivariate (MV) analyses adjusted for sex, age, histology, and stage, loss of PTEN protein expression was associated with significantly shorter disease-free survival (MV hazard ratio: 1.78, 95% confidence interval: 1.01–3.14, $p = 0.048$), whereas no significant associations were seen with *p53* or *KRAS* and epidermal growth factor receptor (*EGFR*) mutation status. Importantly, the prognostic value of absent PTEN staining was limited to adenocarcinomas, with MV disease-free survival hazard ratio of 2.68 (95% confidence interval: 1.35–5.32, $p = 0.005$), whereas no such association was seen in squamous cell carcinomas.

Conclusion: Absence of PTEN protein expression is an independent prognostic marker in early-stage resected lung adenocarcinoma.

Key Words: Phosphatase and tensin homolog, Non–small cell lung carcinoma, Adenocarcinoma, Prognosis.

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Lung cancer is one of the leading causes of cancer death in Western countries.[1] Non–small-cell lung carcinoma (NSCLC) accounts for 70% to 80% of all lung cancers, and comprises several distinct histologic subtypes, the most common being squamous cell carcinoma (SqCC), adenocarcinoma (AdC), and large cell carcinoma. As our understanding of the genetic alterations associated with the development and progression of NSCLC grows, it is now evident that even within a clearly identifiable histologic subtype such as adenocarcinoma, distinct molecular changes may be associated with a spectrum of clinical characteristics that also correlate with disease outcome and response to treatment.[2]

The phosphatase and tensin homolog (*PTEN*) gene on chromosome 10 was cloned in 1997 through its association with the human cancer susceptibility locus at 10q23.[3–6] The PTEN protein functions as a lipid phosphatase, dephosphorylating phosphatidylinositol (3,4,5)-triphosphate, thereby negatively regulating the phosphatidylinositol 3-kinase/AKT (PI3K/AKT) antiapoptotic and proliferation pathway.[7–10] *PTEN* is reported to play a role in apoptosis, cell cycle arrest, cell migration, and metastasis.[10–15] Loss of PTEN protein expression has been linked to

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shorter survival times in patients with NSCLC, tongue cancer, and with more advanced or poorly differentiated tumor in esophageal and oral squamous cell cancers, respectively.[16–18] In addition, PTEN is a key downstream component of the epidermal growth factor receptor (EGFR) pathway.[19] Inhibition of this pathway with EGFR tyrosine kinase inhibitors (TKIs) has been a successful treatment strategy for patients with advanced NSCLC, particularly those whose tumors harbor activating *EGFR* mutations in exons 19 and 21.[20,21] Recent reports suggest that alterations of *PTEN* also are associated with EGFR TKI resistance.[22,23] Therefore, further insights into the role of *PTEN* in NSCLC may provide important prognostic and predictive information especially in tumors that develop resistance to EGFR TKIs.

In this study we conducted a comprehensive analysis to correlate PTEN protein expression with gene copy number, p53 expression, and mutations in the *EGFR* and *KRAS* genes in surgically resected NSCLCs. We also investigated the relationship between *PTEN* status, clinicopathological characteristics, and survival outcomes in these patients.

MATERIALS AND METHODS

Tissue Samples

The study was conducted with an approved protocol from the Research Ethics Board of the University Health Network. Tissue microarrays (TMAs) were constructed using formalin-fixed paraffin embedded archival blocks of patients who underwent surgical resection of their NSCLC at the University Health Network, using the manual tissue arrayer (Beecher Instruments, Silver Spring, MD). Guided by hematoxylin and eosin (H&E)-stained slides, 0.6-mm cores were taken from three areas of high-tumor cellularity, and one from non-neoplastic lung tissue. These cores were arrayed into TMA blocks and DNA was isolated from additional tumor cell rich areas of the same blocks by further coring. Tumor staging was based on the 7th edition of the tumor, lymph node, and the metastasis classification from the International Union Against Cancer.[24] The histological classification was based on the 2004 World Health Organization classification of lung neoplasms.[25] None of these patients received EGFR TKIs before surgical resection.

PTEN Immunohistochemistry Analysis

The PTEN antibody (138G6, Cell Signaling Technology, Danvers, MA) was used and immunohistochemistry (IHC) analysis was carried out on the Ventana Benchmark XT autostainer using the iVIEW DAB detection system for PTEN. Sangale et al.[26] previously confirmed the specificity of this antibody using cell lines with known *PTEN* genotype. We also independently confirmed the specificity in our own laboratory by Western blotting (data not shown). Importantly, the PTEN immunostaining has a built-in control on each section as the tumor stromal fibroblasts and endothelial cells show strongly positive (2+) staining (Fig. 1). Loss of expression is represented by an absence of staining of tumor cell cytoplasm (Fig. 1*A*, *B* versus *D*). Weaker staining that can be distinguished from the background staining level of stromal cells was

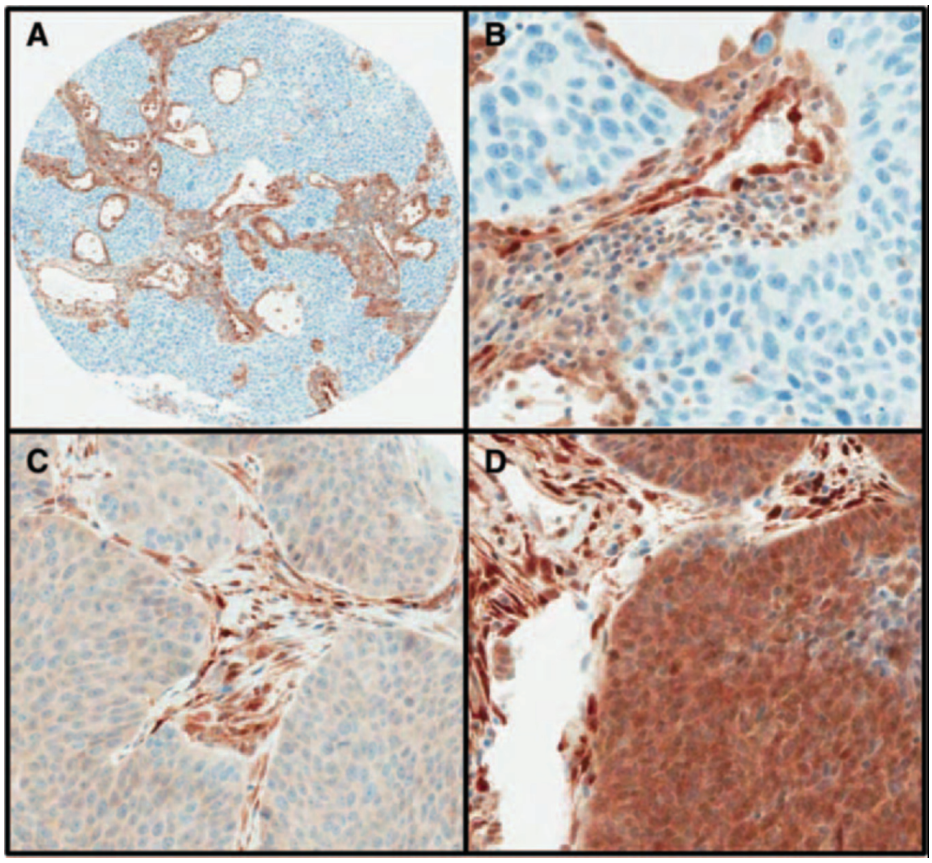

FIGURE 1. Representative images of PTEN immunohistochemistry. ***A***, Low magnification and (***B***) high magnification, tumor showing complete lack of staining for PTEN. ***C***, Low magnification and (***D***) high magnification, tumor showing positive staining for PTEN. PTEN, phosphatase and tensin homolog.

still considered partially positive (1+) for PTEN expression (Fig. 1*C*). Loss of PTEN expression was defined as *complete absence* of cytoplasmic staining in tumor cells. Two pathologists (NY and MAS) evaluated the results independently and discrepant cases were evaluated together with a third pathologist (MST), to reach a final consensus decision.

PTEN FISH Analysis

The fluorescence in situ hybridization (FISH) probes include the Vysis chromosome 10 centromeric probe labeled with Spectrum Aqua (Abbott Laboratories, Abbott Park, IL) and a PTEN probe represented by the bacterial artificial chromosome RP11- 846G17 located at 10q23.31 (purchased from the Center for Applied Genomics Toronto, Canada, labeled with Spectrum Green).

Probe hybridization was performed using standard FISH techniques. Briefly, 5-micron TMA slides were incubated at 56ºC for 24 hours, deparaffinized with xylene, and dehydrated in 100% ethanol. For antigen retrieval, the TMA sections were placed in 10 mM sodium citrate (SSC), pH 6.4, at 80ºC for 50 minutes and rinsed sequentially in SSC and dH_2O. The slides were then digested with 750 U/ml pepsin in 0.01 N HCl solution at 37ºC for 18 minutes, then in dH_2O, pH 7.0, at room temperature for 10 minutes and dehydration in an ascending ethanol series for 2 minutes each. Each probe was then added to the hybridization areas, cover slipped, and sealed with rubber cement. Codenaturation was carried out at 80ºC for 10 minutes in a microprocessor-controlled system (Hybrite; Abbott Laboratories), and the sections were hybridized at 37ºC overnight. Two posthybridization washes consisted of SSC, pH 7.0, containing 0.3% octylphenyl-polyethylene glycol (IGEPAL) for 2 minutes at 72ºC followed by 2 minutes in SSC, pH 7.0, at room temperature. After rinsing in dH_2O, the slides were air dried in a dark chamber; this was followed by nuclear counterstaining with 4´,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).

PTEN copy number was evaluated by dual-color FISH in 100 tumor nuclei with an inhouse *PTEN* probe labeled with Spectrum Green and a Vysis Chromosome Enumerating Probe for chromosome 10 (Abbott Laboratories, Abbott Park, IL) labeled with Spectrum Aqua. As nuclei of NSCLC are known to vary in size with ploidy, a relatively high cutoff of 63% was used to address loss of signal secondary to nuclear truncation.[27,28] This cutoff is equivalent to 2 SD above the mean percent cells with one signal of the PTEN probe and two signals of the control chromosome enumerating probe in appropriate control nuclei.

KRAS and *EGFR* Mutational Profiling and P53 Immmunohistochemistry

KRAS and *EGFR* mutations were analyzed by mass spectrometric profiling for mutational sequence variants using the OncoCarta Panel v1.0 (Sequenom, San Diego, CA), as reported previously.[29] Standard Sanger sequencing methodology verified all identified mutations. p53 Immunohistochemistry was performed using the DO-7 mouse monoclonal antibody (Vector Laboratories, Burlington, Ontario, Canada) using a Ventana Benchmark XT autostainer.

Statistical Analysis

Demographic proportions in alteration of *PTEN* were compared using Fisher's exact test of association for sex, histology, smoking history, p53 immunostaining, as well as *EGFR* and *KRAS* mutation status. The Mann-Whitney test was used to test for differences in age, whereas the Cochran–Armitage test was used to test for trend in stage. Disease-free survival (DFS) was defined as the time from surgery until date of relapse, death, or the last recorded date of follow-up. The Kaplan–Meier method was used to calculate 3-year DFS probabilities, and survival curves were compared with a log-rank test. Variables of interest were tested in the presence of other clinical factors using a Cox proportional hazards model. A two sided p value of 0.05 was considered statistically significant. All statistical analyses were performed using the open-source statistical software R, version 2.12.1.

RESULTS

Tumors from 152 patients who underwent curative surgical resection for primary NSCLC at the University Health

TABLE 1. Clinicopathological Characteristics of Patients Studied

Characteristics	Total Patients = 152
Age	
Median (yrs) (range)	66.9 (42–88)
Sex	
Female	77 (50.7%)
Male	75 (49.3%)
Histology	
Adenocarcinoma	94 (61.8%)
Squamous cell carcinoma	44 (28.9%)
Others	14 (9.2%)
TNM stage	
I	89 (58.6%)
II	29 (19.1%)
III	23 (15.1%)
IV	11 (7.2%)
Smoking	
Yes	120 (78.9%)
No	23 (15.1%)
No data	9 (5.9%)
***EGFR* mutation**	
Mutant	22 (14.5%)
Wild type	130 (85.5%)
***KRAS* mutation**	
Mutant	34 (22.4%)
Wild type	118 (77.6%)
P53 immunohistochemistry	
Positive	68 (44.7%)
Negative	84 (55.3%)

EGFR, epidermal growth factor receptor; TNM, tumor, lymph node, and metastasis.

TABLE 2. Correlation between PTEN Protein Expression by Immunohistochemistry and Gene Copy Changes by Fluorescence in situ Hybridization and Clinicopathological Characteristics

Clinicopathological Characteristics	Protein Expression			Gene Copy Deletion		
	Lost (%)	Retained (%)	*p*	Present (%)	Absent (%)	*p*
PTEN	63 (41)	89 (59)		7 (5.6)	117 (94.4)	
Age (yrs)						
Median (range)	66 (44–84)	68 (42–88)	0.966	67 (47–82)	67 (42–88)	0.94
Sex						
Female	25 (33)	52 (67)	0.032	4 (7)	55 (93)	0.71
Male	38 (51)	37 (49)		3 (5)	62 (95)	
Histology						
AdC	32 (34)	62 (66)	0.021	1 (1)	74 (99)	0.009
SqCC	26 (59)	18 (41)		6 (16)	32 (84)	
Others	5 (36)	9 (64)		0	11 (100)	
TNM stage						
I	33 (37)	56 (63)	0.29	3 (4)	69 (96)	0.66
II	13 (45)	16 (55)		2 (8)	23 (92)	
III	13 (57)	10 (43)		2 (11)	17 (89)	
IV	4 (36)	7 (64)		0	8 (100)	
Smoking						
Yes	56 (47)	64 (53)	0.107	6 (6)	93 (94)	1.0
No	6 (26)	17 (74)		1 (6)	16 (94)	
No data	1 (11)	8 (89)		0	8 (100)	
***EGFR* mutation**						
Yes	6 (27)	16 (73)	0.167	1 (6)	16 (94)	1.0
No	57 (44)	73 (56)		6 (6)	101 (94)	
***KRAS* mutation**						
Yes	14 (41)	20 (59)	1.00	0	25 (100)	0.34
No	49 (42)	69 (58)		7 (7)	92 (93)	
p53 IHC						
Positive Negative	28 (41) 35 (42)	40 (59) 49 (58)	1.00	4 (7) 3 (4)	53 (93) 64 (96)	0.70

AdC, adenocarcinoma; SqCC, squamous cell carcinoma; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; KRAS, ; TNM, tumor, lymph node, and metastasis; PTEN, phosphatase and tensin homolog.

Network from April 2005 to June 2009 were included in this study.[27] Patient characteristics are shown in Table 1. Half the patients were women, there were more adenocarcinomas (61.8%), the majority were smokers (78.9%), and most had stage I disease (58.6%). The distribution of mutations and p53 immunoreactivity was typical for North American NSCLC patients with 22 of 152 (14%) and 34 of 152 (22%) harboring *EGFR* and *KRAS* mutations, respectively.

PTEN Protein Expression

There were 54 and 35 cases with 1+ and 2+ staining (retained expression), respectively. Loss of PTEN expression (completely negative) was seen in 63 of 152 cases (41.4%) (Table 2). PTEN loss was observed more frequently in men than in women (51% versus 33%, $p = 0.032$) and in SqCC than in AdC (59% versus 34%, $p = 0.021$). In patients who were lifetime nonsmokers, PTEN loss was seen in only 26%, but the difference compared with patients with a smoking history was not significant. A similar proportion of patients (27%) with *EGFR* mutations demonstrated PTEN loss. There was no significant correlation between loss of PTEN expression and *KRAS* mutations or p53 immunostaining status.

PTEN Copy Number Changes and Correlation with Protein Expression

FISH results were available for 124 of 152 cases, as some TMA cores failed to provide interpretable results because of loss of tumor-containing cores or poor hybridization. Seven cases (5.6%) demonstrated *PTEN* deletion, including hemizygous loss in four cases, monosomy in two cases, and homozygous loss in one case (Fig. 2). Unexpectedly, there were four cases with increased *PTEN* copy number: one with *PTEN* cluster formation, whereas the other three showed additional *PTEN* copies without *PTEN* cluster formation. All seven *PTEN* deleted cases were negative by PTEN IHC. However, 49 cases without *PTEN* deletion demonstrated loss of PTEN protein expression. Six of the seven cases with *PTEN* deletions

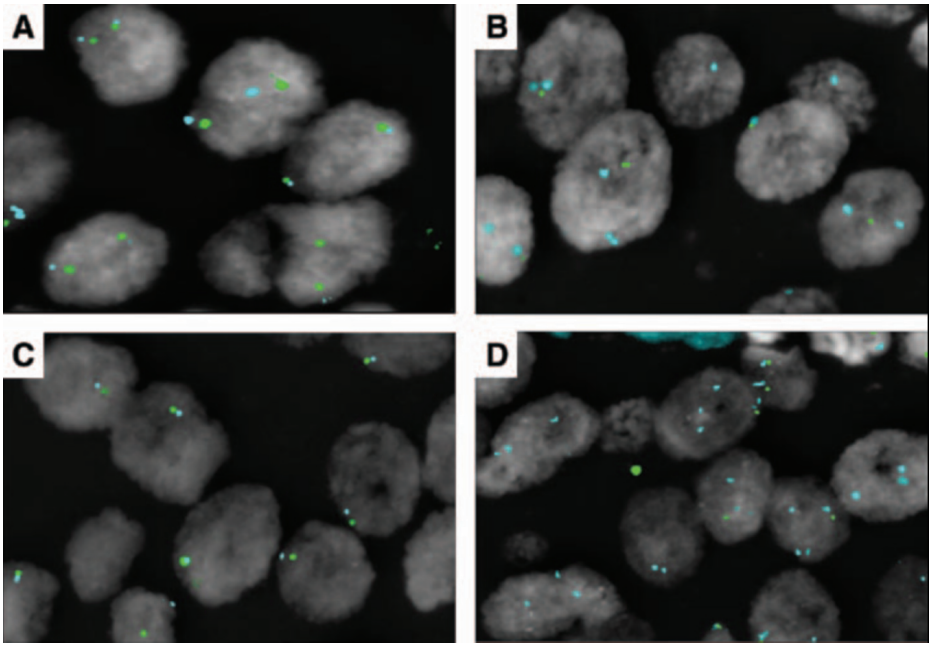

FIGURE 2. Representative photographs (100× magnification) of dual-color fluorescent in situ hybridization of non–small-cell lung tumors using probes for PTEN (green/FITC) and the centromere of chromosome 10 centromeric probe (blue/aqua), with grayscale 6-diamidino-2-phenylindole nuclear stain: ***A***, PTEN diploidy; (***B***), PTEN hemizygous loss as predominant pattern; (***C***), PTEN hemizygous loss with chromosome 10 monosomy as predominant pattern; (***D***), PTEN homozygous loss as predominant pattern. PTEN, phosphatase and tensin homolog.

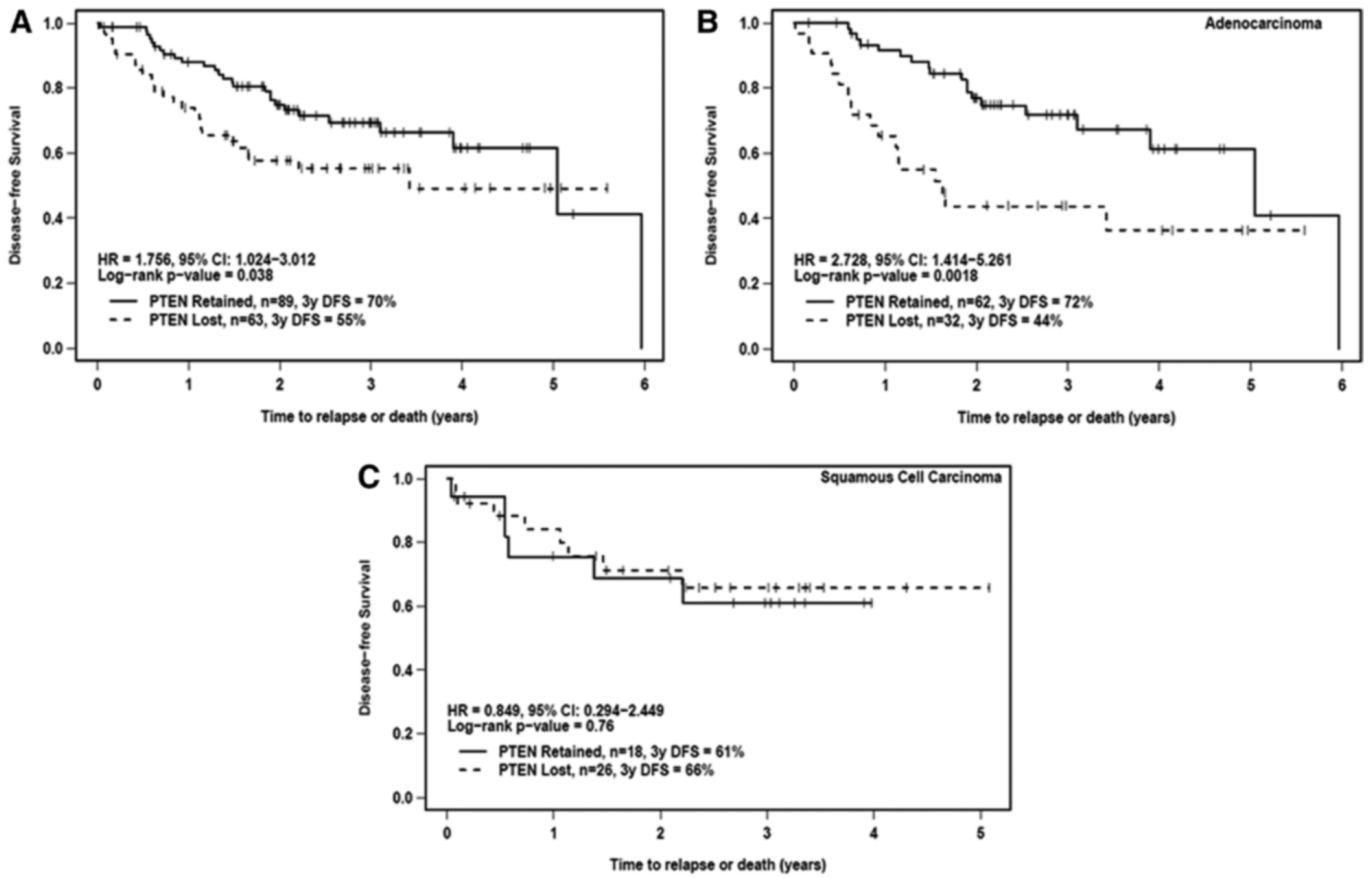

FIGURE 3. Kaplan–Meier DFS curves based on PTEN immunohistochemistry status. ***A***, All patients based on PTEN status showing PTEN negative tumors were associated with poorer DFS compared with PTEN positive tumors. Adenocarcinomas (B) and (***C***) squamous cell carcinoma, according to PTEN status demonstrating significant differences in DFS based on histology. PTEN, phosphatase and tensin homolog; DFS, disease-free survival; CI, confidence interval; HR, hazard ratio.

TABLE 3. Correlation of Disease-Free Survival with PTEN Protein Expression and Other Clinical, Pathological, and Molecular Factors

	Hazard Ratio	95% Confidence Interval	Wald *p*
Univariate Analysis			
Sex (male vs. female)	1.04	0.61–1.79	0.88
Age (≥65 vs. 65 yrs)	0.87	0.51–1.51	0.63
Histology (AdC vs. others)	1.17	0.65–2.08	0.60
Stage (III/IV vs. I/II)	2.42	1.37–4.25	0.0022
PTEN immunohistochemistry (− vs. +)	1.76	1.02–3.01	0.041
EGFR mutation (mutant vs. wild type)	0.99	0.47–2.11	0.98
KRAS mutation (mutant vs. wild type)	1.57	0.87–2.83	0.13
p53 immunohistochemistry (+ vs. −)	1.24	0.72–2.14	0.43
Multivariate analysis (Total)			
PTEN staining (− vs. +)	1.78	1.01–3.14	0.048
Sex (male vs. female)	1.05	0.59–1.84	0.88
Age	0.99	0.97–1.02	0.69
Histology (AdC vs. others)	1.12	0.60–2.09	0.72
Stage (III/IV vs. I/II)	2.32	1.29–4.20	0.005
Multivariate analysis (Adenocarcinoma only)			
PTEN staining (− vs. +)	2.68	1.35–5.32	0.005
Sex (male vs. female)	0.74	0.37–1.47	0.39
Stage (III/IV vs. I/II)	2.42	1.24–4.72	0.009

PTEN, phosphatase and tensin homolog; −, loss of expression; +, retained expression; AdC, Adenocarcinoma; EGFR, epidermal growth factor receptor; KRAS,

occurred in SqCC cases, accounting for 35.3% (6 of 17) of the PTEN-IHC–negative cases. In contrast, *PTEN* deletion was rare (1 of 75) in AdC cases.

Correlation between PTEN Protein Expression, PTEN Copy Number, and Survival

To determine the impact of PTEN status on clinical outcome, we assessed DFS for the 152 patients included in the IHC study. We initially correlated PTEN expression with survival according to the intensity of positive staining (1+, 2+); however, we found no significant differences. Therefore we focused on comparisons between any intensity of staining (1+ and 2+) and complete absence of staining. The median follow-up time was 2.38 years (range, 0.07– 5.97 years). In univariate analysis, stage was a significant prognostic marker for DFS, but not age, sex, or histology. Patients with loss of PTEN expression had significantly worse outcome than those with retained PTEN expression (hazard ratio [HR] 1.76, 95% confidence interval [CI]: 1.02–3.01, log-rank $p = 0.038$; Fig. 3*A*). In contrast, *KRAS*, *EGFR*, and p53 were not significantly prognostic (Table 3). The prognostic effect was, however, significantly different when stratified by histology. In patients with adenocarcinoma, PTEN loss was associated with significantly shorter DFS (HR 2.72, 95% CI: 1.41–5.26, log-rank $p = 0.002$; Fig. 3*B*), whereas no differences were seen in squamous cell histology (Fig. 3*C*). In multivariate analysis adjusted for sex, age, histology, and stage (Table 3), loss of PTEN expression remained not only significant for shorter DFS in all histologies (HR 1.78, 95% CI: 1.01–3.14, $p = 0.048$), but also for significantly shorter DFS in AdC only (HR 2.68, 95% CI: 1.35–5.32, $p = 0.005$). Because of the very low number ($n = 7$) of *PTEN* deleted cases, correlation with survival outcome was not performed.

DISCUSSION

In the present study, we found that 41% of NSCLCs tested had loss of PTEN protein expression by IHC. Although the lack of PTEN expression was significantly more frequent in squamous cell carcinoma, it was only prognostic in patients with adenocarcinoma. Also, although *PTEN* deletions detected by FISH correlated with absent expression by IHC, these deletions were only detected in seven cases and most of these were squamous cell carcinomas.

The inactivation of *PTEN* frequently is found in a variety of tumors, including lung, endometrial, bladder, renal, and breast cancers.[6] Absent PTEN expression has been reported to occur in 24% to 78% of NSCLC (Table 4). Among previously published studies,[10,19,32–37] only two[34,36] reported a significantly greater prevalence in SqCC (or non-AdC) than AdC (Table 4), similar to what we have observed. In both of these studies and our own, a Cell Signaling antibody was used. However, only Scrima et al.[36] identified the antibody as clone 138G6, the same clone used in this study. Antibody specificity has always been a poorly controlled factor in IHC studies. In the case of PTEN, Sangela et al.[26] reported previously that the 138G6 antibody was the most specific among the 11 antibodies that they evaluated. The greater frequency of PTEN loss of expression in men than in women could be related to the differential expression in SqCC versus AdC. In our study, there was no relationship between tumor stage and PTEN expression, in contrast to two other studies that suggested some correlation, yet reporting a contradictory relationship.[10,19]

In univariate analysis, patients with loss of PTEN expression had significantly worse outcome than those with retained PTEN expression (HR 1.76, 95% CI: 1.02–3.01, $p = 0.038$), and this association remained significant even after adjusting for other prognostic variables (HR 1.78, 95% CI: 1.01–3.14, $p = 0.048$). Interestingly, when we investigated survival by histology, the poorer DFS associated with PTEN loss was only seen in AdCs. Table 4 also summarizes the literature correlating histology and survival to PTEN expression in NSCLC. Among seven studies that included survival outcome correlation with PTEN expression, six[19,32–35,37] reported a significantly poorer prognosis for patients whose tumors showed absent or low PTEN expression. There is, however, a lack of uniformity in the cutoff chosen for survival correlation, with many studies including low percentage or faint staining in their *negative* cases. Our results demonstrated that when using the Cell Signaling antibody clone 138G6, no difference in DFS was seen between faint PTEN (1+) and stronger PTEN staining intensity (2+). With this approach, the frequencies of negative cases are similar to ours, ranging from 24% to 65% of NSCLC.[19,32,33,37] Other than

TABLE 4. Reported Prevalence and Prognostic Significance of PTEN Expression Loss in Non–small-cell lung cancer

Author (Yr)	Antibody Clone (Source)	Cutoff Negative (−)	Total Number	% PTEN Negative	AdC Studied	% PTEN negative	SqCC Studied	% PTEN Negative	Frequency by Histology	Univariate Prognostic Value (− vs +)	Multivariate Analysis
Soria[37](2002)	(Zymed)	Absence (0)	125	24	75	24	50	24	NS	5-year OS: NS	NA
Marsit[10](2005)	6H2.1 (Cascade)	<50% (lack or reduced)	117	44	64	77%	40	70	NS	NS	NA
Tang[19](2006)	(Beijing Zhong Shan Biotech)	Faint in <20%	102	46	51	39	51	53	NS	5-year OS: p <0.001	NA
Wang[32](2009)	sc-56205 (Santa Cruz)	<5%	172	41	172	41	NA	NA	NA	OS: HR = 1.67, 95% CI 1.06–2.86, p = 0.002	HR = 2.11, 95% CI: 1.70–2.61, p < 0.001 (AdC)
Wang[33](2011)	PAD: PN 37 (Zymed)	Absence (0)	78	65	34	68	44	64	NS	Median progression-free survival p < 0.05	HR=3.07, p = 0.01 (all)
Kaira[34](2011)	(Cell Signaling)	<25%	160	78	106	71	54 (Non- AdC)	93	Non-AdC > AdC	5-year OS: p = 0.009 (all); p = 0.021 (AdC)	NA
Kim[35](2012)	Y184 (Epitomics)	Weak in < 1/3	245	45	91	37	154	49	NS	OS: p = 0.015	HR = 1.91, 95% CI: 0.91–4.02, p = 0.086 (AdC only)
Scrima[36](2012)	138G6 (Cell Signaling)	<25%	104	39	51	27	40	55	SqCC >AdC	NA	NA
Current Study (2012)	138G6 (Cell Signaling)	Absence (0)	152	41	94	34	44	59	SqCC >AdC	3-yr DFS: HR = 1.76, 95% CI: 1.02–3.01, p = 0.038 (all); HR = 2.73, 95% CI: 1.41–5.26, p = 0.002 (AdC)	HR = 1.78, 95% CI: 1.01–3.14, p = 0.048 (all); HR = 2.68, 95%CI: 1.35–5.32, p = 0.005 (AdC only)

SqCC, squamous cell carcinoma; AdC, adenocarcinoma; NA, not available; OS, overall survival; CI, confidence interval; DFS, disease-free survival; NS, not significant; PTEN, phosphatase and tensin homolog; HR, hazard ratio.

our own, one study[33] reported low/absent PTEN expression as a significant independent prognostic marker in NSCLC patients. Our results further suggest that the differences in survival may be driven by the loss of PTEN in AdCs, as its prognostic significance was not apparent among the SqCC patients. A similar result was obtained by Wang et al.,[32] whose study cohort included only AdC patients.

The role of *PTEN* in these tumors seems functionally important to oncogenesis as its loss results in the dysregulation of Akt-dependent and Akt-independent pathways that are known to be critical for the progression of malignant cancers.[38] The PI3K/AKT pathway has been implicated in tumorigenesis because of its role in cell survival and metabolism. The loss of PTEN expression may be induced by a variety of mechanisms, including homozygous deletion, nonsense mutation with loss of heterozygosity, and promoter methylation.[37–39] Indeed, *PTEN* promoter methylation has been reported in a series of 132 NSCLC patients to be independently associated with poorer prognosis when compared with unmethylated samples.[38] It is recognized that mutation and homozygous deletion of *PTEN* in lung cancer are uncommon events, whereas the frequency of hypermethylation of *PTEN* promoter is variable (0–35%).[10,37–40] In our series, only seven cases were *PTEN* deleted by FISH and all seven cases showed loss of PTEN protein expression. Although *PTEN* copy number change may be a cause of *PTEN* inactivation in some patients, our study suggests that additional mechanisms for loss of PTEN protein expression in patients without copy number change are also important. Marsit et al.[10] suggest that regulation of PTEN is not always at the genetic level but also may occur at the transcriptional or translational level, which may also explain the observed limited correlation between gene copy number and IHC.

Approximately 70% of tumors with *EGFR*-activating mutations respond to *EGFR* TKIs.[41] However, some tumors demonstrate primary resistance to TKIs, and most will develop secondary resistance at some time point. The T790M *EGFR* mutation and amplification of *MET* are both recognized mechanisms of resistance.[42,43] Recently, Yamamoto et al.[23] reported that loss of PTEN expression contributes to gefitinib and erlotinib resistance in NSCLC, and Sos et al.[22] reported a novel resistance mechanism in *EGFR*-mutant NSCLC involving PTEN loss. Furthermore, a recent retrospective study investigating the association of *PTEN*, *PIK3CA*, and *EGFR* copy number with responses to gefitinib, found that a combination of *PTEN* loss, PIK3CA gain, and EGFR copy number was associated with limited responses, especially in EGFR wild-type tumors.[44] In our study, 22 of 152 cases harbored *EGFR* mutations, and six of these also demonstrated loss of PTEN by IHC. As these patients did not receive EGFR TKIs in the adjuvant setting, we do not have information on the sensitivity of these tumors to EGFR TKI therapy, although the abovementioned studies suggest that primary resistance might occur in these tumors.

Currently, there are several active early-phase clinical trials evaluating PI3K inhibitors either alone or in combination with chemotherapy, EGFR TKIs, or monoclonal antibodies. Preliminary data suggest that such agents are effective

in tumors in which the PI3K-AKT pathway is activated, as occurs with mutations or gene amplification in the *PIK3CA* gene. *PIK3CA* mutations are uncommon but have been identified in 2% to 4% of primary NSCLC,[45,46] predominantly in exons 9 and 20. As loss of PTEN results putatively in the constitutive activation of the PI3K pathway, and this occurs more frequently than *PIK3CA* mutation/amplification, it is possible that more NSCLC and particularly AdCs may be susceptible to abrogation of the PI3K-AKT and that it may be advisable also to select patients by identifying the PTEN status rather than focusing purely on *PIK3CA* mutations or phosphorylated AKT.

In conclusion, we have demonstrated that although loss of PTEN protein expression occurs significantly more frequently in squamous cell histology, it exerts a significant effect on DFS only in AdCs. This histological association may explain the discrepancies in PTEN results previously reported in NSCLC. Contrary to other studies, we did not find an association with p53 staining, nor with more advanced stage. The poorer DFS observed with PTEN loss was independent of *EGFR* and *KRAS* mutation status. Reasons for the observed prognostic differences associated with PTEN loss in AdC but not squamous cell carcinoma warrant further investigation.

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